TROUBLESHOOTING TIPS - ELISA

Positive results in negative control

Contamination of reagents/samples

May be contamination of reagents or samples, or cross contamination from splashing between wells. Use fresh reagents and pipette carefully.

Sandwich ELISA – Detection antibody is detecting coating antibody.

Check the correct coating antibody and detection antibodies are being used and that they will not detect each other.

Insufficient washing of plates

Ensure wells area washed adequately by filling the wells with wash buffer. Ensure all residual antibody solutions are removed before washing.

To much antibody used leading to non-specific binding

Check the recommended amount of antibody suggested. Try using less antibody.

High background across entire plate

Conjugate to strong or left on too long

Check dilution of conjugate, use it at the recommended dilution. Stop the reaction using stop buffer as soon as the plate has developed enough for absorbance readings.

Substrate solution or stop solution is not fresh

Use fresh substrate solution. Stop solution should be clear (if it has gone yellow, this is a sign of contamination and it should be replaced).

Reaction not stopped

Colour will keep developing if the substrate reaction is not stopped.

Plate left too long before reading on the plate reader

Colour will keep developing (though at a slower rate if stop solution has been added)

Contaminants from laboratory glassware

Ensure reagents are fresh and prepared in clean glassware. Sterilise glassware beforehand if

Substrate incubation carried out in the light
Substrate incubation should be carried out in the dark.

Incubation temperature too high

Antibodies will have optimum binding activity at the correct temperature. Ensure the incubations are carried out at the correct temperature and that incubators are set at the correct temperature and working. Incubation temperature may require some optimisation.

Non-specific binding of antibody.

Ensure a block step is included and a suitable blocking buffer is being used. We recommend using 5 to 10% serum from the same species of the secondary antibody, or bovine serum.
Ensure wells are pre-processed to prevent non specific attachment
Use an affinity purified antibody, preferably pre-absorbed

Also check suggestions listed under 'Positive results in negative control'

Low absorbance values

Target protein not expressed in sample used/ Low level of target protein expression in sample used

Check the expression profile of the target protein to ensure it will be expressed in your samples. If there is low level of target protein expression, increase the amount of sample used, or you may need to change to a more sensitive assay. Ensure you are using a positive control within the detection range of the assay.

Insufficient antibody

Check the recommended amount of antibody is being used. The concentration of antibody may require increasing for optimisation of results

Substrate solutions not fresh or combined incorrectly

Prepare the substrate solutions immediately before use. Ensure the stock solutions are in date and have been stored correctly, and are being used at the correct concentration. Ensure the reagents are used as directed at the correct concentration.

Reagents not fresh or not at the correct pH

Ensure reagents have been prepared correctly and are in date

Incubation time not long enough

Ensure you are incubating the antibody for the recommended amount of time, if an incubation time is suggested. The incubation time may require increasing for optimisation of results.

Incubation temperature too low

Antibodies will have optimum binding activity at the correct temperature. Ensure the incubations are carried out at the correct temperature and that incubators are set at the correct temperature and working. Incubation temperature may require some optimisation.

Ensure all reagents are at room temperature before proceeding.

Stop solution not added

Addition of stop solution increases the intensity of colour reaction and stabilizes the final colour reaction

High absorbance values for samples and/or positive control - absorbance does not go down as the sample is diluted down the plate).

The concentration of samples or positive control is too high and out of range for the sensitivity of the assay. Re-assess the assay you are using OR reduce the concentration of samples and control by dilution before adding to the plate. Take the dilution into account when calculating the resulting concentrations.

Inconsistent absorbances across the plate

Plates stacked during incubations

Stacking of plates does not allow even distribution of temperature across the wells of the plates. Avoid stacking.

Pipetting inconsistent

Ensure pipettes are working correctly and are calibrated. Ensure pipette tips are pushed on far enough to create a good seal. Take particular care when diluting down the plate and watch to make sure the pipette tips are all picking up and releasing the correct amount of liquid.
This will greatly affect consistency of results between duplicates.

Antibody dilutions/Reagents not well mixed

To ensure a consistent concentration across all wells, ensure all reagents and samples are mixed before pipetting onto the plate.

Wells allowed to dry out

Ensure lids are left on the plates at all times when incubating. Place a humidifying water tray (bottled clean/sterile water) in the bottom of the incubator.

Inadequate washing

This will lead to some wells not being washed as well as others, leaving different amounts of unbound antibody behind which will give inconsistent results.

Bottom of the plate is dirty affecting absorbance readings.

Clean the bottom of the plate carefully before re-reading the plate

Colour developing slowly

Plates are not at the correct temperature

Ensure plates are at room temperature and that the reagents are at room temperature before use

Conjugate too weak

Prepare the substrate solutions immediately before use. Ensure the stock solutions are in date and have been stored correctly, and are being used at the correct concentration. Ensure the reagents are used as directed, at the correct concentration.

Contamination of solutions

*Presence of contaminants, such as sodium azide and peroxidise can affect the substrate reaction. Avoid using reagents containing these preservatives***.**